Tenascin-C deficiency impairs alveolarization and microvascular maturation during postnatal lung development

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Running title. Tenascin-C deficiency impairs pulmonary alveolarization

Abbreviations.

TNC	Tenascin-C
WT	Wildtype
2D	Two-dimensional
3D	Three-dimensional
TUNEL	TdT-mediated dUTP nick end labeling

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Abstract

After the airways have been formed by branching morphogenesis the gas-exchange area of the developing lung is enlarged by the formation of new alveolar septa (alveolarization). The septa themselves mature by a reduction of their double layered capillary networks to single layered ones (microvascular maturation). Alveolarization in mice is subdivided into a first phase (postnatal days 4-21, classical alveolarization), where new septa are lifted off from immature pre-existing septa, and a second phase (days 14-adulthood, continued alveolarization), where new septa are formed from mature septa. Tenascin-C (TNC) is a multi-domain extracellular matrix protein contributing to organogenesis and tumorigenesis. It is highly expressed during classical alveolarization, but afterwards it is markedly reduced. To study the effect of TNC deficiency on postnatal lung development, the formation and maturation of the alveolar septa was followed stereologically. Furthermore, the number of proliferating (Ki-67-positive) and TUNEL-positive cells was estimated. In TNC deficient mice for both phases of alveolarization a delay and catch-up was observed. Cell proliferation was increased at days 4 and 6, at day 7 thick septa with an accumulation of capillaries and cells were observed, and the number of TUNEL-positive cells (dying cells or DNA-repair) was increased at day 10. While at days 15 and 21 premature microvascular maturation was detected, the microvasculature was less mature at day 60 as compared to wildtype. No differences were observed in adulthood. We conclude that TNC contributes to the formation of new septa, to microvascular maturation, and to cell proliferation and migration during postnatal lung development.

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New & Noteworthy

Previously we showed that the extracellular matrix protein tenascin-C takes part in prenatal lung development by controlling branching morphogenesis. Now we report that tenascin-C is also important during postnatal lung development, because tenascin-C deficiency delays the formation and maturation of the alveolar septa during classical, but also during continued alveolarization. Adult lungs are undistinguishable from wildtype due to a catch-up formation of new septa.

Introduction

Structural lung development

Starting from the lung buds (lung anlage) the conducting airways and parts of the respiratory airways are formed by continuous cycles of branching and growth into the surrounding mesenchyme (**branching morphogenesis** (65, 83)). During **the stage of alveolarization** the gas exchange surface area is enlarged by the lifting off of new alveolar septa from the pre-existing septa. The newly formed septa increase in height and subdivide the existing airspaces into smaller units (**septation**), called alveoli. During the lifting off of new alveolar septa one leaflet of the existing double layered capillary network within the existing septa folds up and gives rise to a new double layered capillary network inside the newly formed septa (3, 6, 7, 65, 87). Depending on the point of view, this process is either call alveolarization or septation. While alveolarization focuses on the formation of new airspaces (alveoli), septation focuses on the formation of new walls (septa) which are subdividing the existing airspaces. In order to optimize the gas exchange the double layered capillary networks of all septa are reduced to a central, single layered one by capillary fusion and the former central layer of connective tissue is reduced to a thin fibrous meshwork interwoven with the capillaries (**microvascular maturation**). Alveolarization and microvascular maturation start in parallel around postnatal day 4 in mice and rats and continue until young adulthood (50, 59, 65, 70). The stage of alveolarization can be subdivided into two phases. **Classical alveolarization** (days 4-21 in mice and rats) is characterized by the lifting off/folding up of new septa from immature pre-existing septa containing a double layered capillary network, while during **continued alveolarization** (day 14-adulthood (roughly days 36-60) in mice and rats) new septa are lifted off/folding up of mature pre-existing septa containing a single layered capillary network (1, 50, 70). In humans alveolarization is considered to start before birth and last up to young adulthood, and microvascular maturation is regarded to last until 2-3 years of age (65, 66, 83).

Cell proliferation and programmed cell death

While the structural mechanism of alveolarization/septation has been well established, the knowledge about the cellular processes and molecular signals guiding the lifting off of new septa is still limited (61, 67). It is well recognized that smooth muscle cells, elastic fibers, and collagen fibrils appear concentrated at the free edges of the existing and newly forming septa throughout septation. The presence of these three components seems to be crucial for the formation of new alveolar septa (20, 61, 67). In addition, the importance of cell proliferation for the lifting off of new septa was emphasized by the finding that in rats the rate of cell proliferation of all major cell types increased exactly in parallel to the beginning of classical alveolarization.(39, 45). The absolute number of fibroblasts and epithelial cells is later diminished by apoptosis. In rats a peak of programmed cell death was detected at the end of the third postnatal week (45, 68). To the best of our knowledge programmed cell death has so far never been followed during postnatal lung development in mice, but is expected to be similar.

Tenascin-C

It is well acknowledged that the extracellular matrix plays an important role in regulating the behavior of cells that contact it. During prenatal lung development there is much evidence that different components of the extracellular matrix, such as tenascin-C elastin, fibronectin and different laminin-isoforms have unique functions in the regulation of branching morphogenesis (61). However, less is known about the role of the extracellular matrix on alveolarization and microvascular maturation. Tenascin-C (TNC) is a large, hexameric glycoprotein of the extracellular matrix. It is transiently expressed during organogenesis, where it is especially prominent at mesenchymal-epithelial interaction sites and along pathways of migrating cells. TNC is markedly reduced in adult tissues, but reappears under pathological conditions such as inflammation and tumorigenesis (8, 9, 21, 23, 61, 78, 84). During prenatal lung development TNC accumulates in the basement membranes and mesenchyme surrounding the branching and growing tips of the bronchial tree (41, 85, 88).

118 At this location it contributes to the control of branching morphogenesis (60). At the
119 beginning of classical alveolarization TNC appears concentrated at the tips of the newly
120 forming septa in parallel to smooth muscle cells, elastic fibers, and collagen fibrils. During the
121 following postnatal lung development and during adolescence TNC expression declines to
122 rarely detectable levels (38, 53, 62, 85). TNC expression is up-regulated by different growth
123 factors, cytokines, as well as by mechanical stress (8, 15). It was shown to be down
124 regulated by glucocorticoids (36, 58) and also in surgical induced congenital diaphragmatic
125 hernia (81).

126 Antibody perturbation assays and tissue culture studies have suggested multiple
127 functions for TNC (12). TNC has been shown to inhibit adhesion to fibronectin of most cells
128 in culture, but for some cells, it functions as adhesion substrate. Therefore, it has been
129 classified as adhesion-modulating protein (10, 54, 63). Furthermore, depending on the cell
130 type TNC has been demonstrated to promote or inhibit cell migration and cell proliferation,
131 and to modulate cell shape (36, 46) (18). Given the multiplicity of functions which have been
132 suggested for TNC by in vitro studies it was rather surprising when TNC null mice were
133 initially reported to show no abnormalities. At the same token no mechanism of
134 compensation for the loss of TNC was found (5, 18, 24, 47, 62, 80). Looking in more detail it
135 became evident that TNC knockout mice show subtle phenotypes, e.g. [i] behavioral
136 abnormalities (26, 40), [ii] a reduced hematopoietic activity of bone marrow cells (52), [iii] an
137 impaired healing of corneal wounds which were exposed to mechanical stress (48), [iv] a
138 suppression of the formation of fibrous adhesions after injury of temporomandibular joint
139 (73), [v] a reduced Wnt/β-catenin signaling combined with a reduced proliferation and
140 migration of stem cells in whisker follicle stem cell niches (32, 79).

141 Developmental alterations have also been reported (14). E.g., [i] fetal lung organ
142 cultures of TNC-null mice showed a reduction of the number of branches, while the growth of
143 the lung explants was not altered (60), and [ii] an increased migration and reduced
144 proliferation of neural precursor cells was detected during the development of the central
145 nervous system (40).

Aim of the present study

Although it is well recognized that in the developing lung the expression of TNC peaks at the start of the first phase of alveolarization (classical alveolarization), the effect of TNC deficiency during this phase has never been investigated so far. The aim of the present study was to provide this information. Therefore, we followed alveolarization/septation and microvascular maturation in the TNC null mice strain generated by Forsberg et al. (24) and in matched wildtype mice using morphological and stereological methods. In addition, the extent of cell proliferation and of TUNEL-positive cells was compared between TNC null and wildtype lungs.

Material and methods

Animals and tissues

Lungs from the TNC null mouse strain “Tnc tm1Ref” of Forsberg et al. (24) and from 129/SV wildtype control animals were obtained between postnatal days 2-86 as described in the following. For every data point an N = 3-8 male animals was used (see figure legends), because the experiments were done at a time were the ethics committee asked for one sex only in order to reduce the number of animals necessary for the study. The animals were housed in the central animal facility of the University of Bern at a 12/12 hour day/night circle. They received water and food ad libitum. The animals were deeply anesthetized using a mixture of medetomidin, midazolam, and fentanyl (22) and afterwards euthanized by exsanguination during the removal of the lung. After abdomen and thorax of the deeply anesthetized mice were opened, the airspace was filled via tracheal installation with freshly prepared 4% paraformaldehyde in PBS (10mM sodium phosphate, containing 127 mM sodium chloride, pH 7.4) at a constant pressure of 20 cm water column. At this pressure, the lung reaches roughly its total lung capacity. In order to prevent a recoiling of the lung, the pressure was maintained at least for 2h at 4°C. For the immunohistochemical investigation, the pulmonary blood vessels were beforehand perfused with phosphate-buffered saline (PBS, 10 mM sodium phosphate, containing 127 mM sodium chloride, pH 7.4), containing 5 U/ml heparin, 10 mg/ml procaine, and 10 mM EDTA (Fluka Chemie AG, Buchs, Switzerland).

Handling of the animals before and during the experiments, as well as the experiments themselves, were approved and supervised by the Swiss Agency for Environment, Forests and Landscape and the Veterinary Service of the Canton of Berne. For ethical reasons we were obliged to keep the number of animals as low as possible. Therefore, we used the left lung for the stereological studies, the right lower lobe for imaging, and the remaining lobes of histochemical staining. According to Zeltner et al. and Barré et al. (4, 86) the lobes represent a representative sample of the entire lung.

For light microscopical morphometry as well as for TdT-mediated dUTP nick end labeling assay and Ki-67-staining the left lung was dehydrated en bloc in a graded series of

ethanol and embedded in paraffin using Histoclear™ (Life Science International, Frankfurt, Germany) as intermedium. A series of step sections of 4.5 µm thickness was obtained perpendicular to the longitudinal axis of the left lung at 10-13 equally spaced locations. The gap between the locations (length of the step) was constant for all lobes obtained at the same postnatal day, but increased with the size of the lobes. The first location was determined as follows. The blocks were cut until first pieces of lung appeared in the sections. Afterwards a randomly selected number of sections was discarded before the first step section was taken / the first location was reached. This number was smaller than the number of sections between two equally spaced locations. The sections were transferred onto silanized micro slides and air-dried overnight at 37°C. Sections used for light microscopical morphometry were stained with fuchsine.

Approximately 40 images were taken from all serial sections of the left lung of each animal according to a systematic random sampling scheme (19). Images were recorded using a Leica DM RB light microscope (Glattbrugg, Switzerland) equipped with a motorized Maerzheuser XY stage (Wetzlar, Germany) and a JVC 930 3-chip color video camera (Oberwil, Switzerland) and the software analySIS (Münster, Germany). The estimation of the volume density of the lung parenchyma, the septal surface area density, the length of the free septal edge, as well as the number of TUNEL-positive cells was done at a final magnification of 250x, whereas for the estimation of the number of proliferating cells and the total number of cells a final magnification of 870x was used.

For transmission electron microscopy and synchrotron-radiation x-ray-tomographic microscopy the right upper and right lower lobes were diced into tissue cubes of about 2mm edge length. The tissue blocks were postfixed with 2.5% glutaraldehyde in 0.03M potassium phosphate buffer (pH 7.4, osmolarity 360 mOsm) for at least 48h at 4°C, stained for 1 hour in 1% Na-cacodylate buffered osmium tetroxide solution (osmolarity 350 mOsm, pH 7,4) and stained for another 2 hours in 0.5% uranyl acetate solution. After dehydration in a graded series of ethanol the tissue blocks were embedded in Epon 812 (68).

For transmission electron microscopy 5 Epon embedded tissue blocks of the right upper lobes were randomly taken and ultrathin sections (80-90nm) were cut using a Reichert-Jung Ultracut microtome. Sections were double stained with lead citrate (56) and uranyl acetate (25). One section per block was viewed in a Philips 400 transmission electron microscope. Approximately 25 images per section were taken according to a systematic random sampling scheme (19) by a Morada camera (soft-imaging-system, Münster, Germany) and the software item (Münster, Germany). Stereological measurements were done at a final magnification of 3400x.

For synchrotron-radiation x-ray-tomographic microscopy 5 blocks of the right lower lobes were randomly taken, shaped down to rods of a diameter of 1.3 mm on a watchmaker's lathe and glued on a rod-like holder of a diameter of 3.0 mm. Special care was taken that they were mounted perpendicularly to the surface of the holder in order to fit exactly into the window of the camera.

Immunohistochemistry

Immunohistochemistry was applied to stain proliferating cells with anti-Ki-67, a marker for cell proliferation (71), and to stain TUNEL-positive cells performing the TdT-mediated dUTP nick end labeling assay adapted from Gavrieli and associates (29).

Anti-Ki-67 staining. As described in (68, 69), paraffin sections were cooked in a household pressure cooker in Target Retrieval Solution (DAKO, Glostrup, Denmark) for 13 min at 2 bar, blocked with TBS containing 100mg/ml Casein (Sigma) and incubated over night at 4°C with the monoclonal rat anti-mouse-Ki-67-antibody (Clone Tec-3, DAKO, diluted 1:50 in antibody diluent, DAKO). Immunoreactivity was detected using the biotinylated polyclonal rabbit anti rat antibody (DAKO, diluted 1:200 in antibody diluent, DAKO), streptavidin-biotin horseradish peroxidase complex (DAKO), and 3-amino-9-ethylcarbazole (Sigma) as a substrate. The nuclei were counterstained with Mayer`s hematoxylin (VWR, Darmstadt, Germany).

238 **TUNEL assay**. As described in (68, 69), paraffin sections were digested with 3.6
239 μg/ml proteinase K (21°C, 10 min) and incubated with terminal transferase reaction solution,
240 containing 9 mM digoxigenin-11-dUTP and 0.165 U/ml enzyme (Roche, Rotkreuz,
241 Switzerland) for 40 min at 37°C. The incorporated digoxigenin was detected using an
242 alkaline-phophatase labelled anti-digoxigenin antibody (Roche Rotkreuz, Switzerland; diluted
243 1:1000 in blocking reagent for nucleic acid hybridization and detection, Roche Rotkreuz,
244 Switzerland) and 4-nitro-blue-tetrazolium-chloride (Roche Diagnostics, Mannheim,
245 Germany).
246 Negative controls were performed with nonspecific mouse IgG (Ki-67 staining) or by
247 omitting of the terminal transferase reaction solution (TUNEL). None or only little nonspecific
248 background was observed in all negative controls. In addition, the Ki-67 was observed as a
249 nuclear staining, only.
250
251 **Light microscopical morphometry**
252 After the fixation the volumes of the left lungs were first measured by water
253 displacement (64). After embedding in paraffin and sectioning the lung volumes were
254 estimated by the Cavalieri method (33, 49). Both volumes were used to calculate the
255 shrinkage for every lung in order to correct for the shrinkage. The volume density of the lung
256 parenchyma (airspaces and septal tissue, excluding bronchi, bronchioli and blood vessels >
257 20μm in diameter) was estimated by point counting.
258 The surface density of the alveolar septa was estimated by intersection counting. The
259 absolute values were calculated as the product of the surface density and the lung volume
260 for each animal and each time-point (33, 82).
261 The length density and length of the free septal edge was estimated stereologically as
262 described and applied by Schittny and coworkers (50, 58, 70, 77). Briefly, this approach is
263 based on the following two principles. First, any length appearing in three-dimensional (3D)
264 space may be stereologically estimated by counting the number of points cutting the plane of
265 2D sections (33, 82). Second, in 3D space every airspace possesses one entrance ring

266 which is represented by the free edges of the alveolar septa. Because the free septal edges
267 are recognized as tips of the cut septa in 2D sections, their length density was estimated by
268 counting the number of the tips of the cut septa in a reference area on paraffin sections. By
269 simple enlargement of the lung, without the addition of new septa, the length density of the
270 free edges of the alveolar septa will decrease, because a volume increases by a factor of x^3,
271 while a length increases only by a factor of x^1. This kind of growth follows the principle of
272 isometric scaling and geometric similarity – meaning that proportional relationships are
273 preserved. E.g., when the volume increases by a factor of 8, the surface increases by a
274 factor of 4 and the length by a factor of 2. This principle, the square-cube law, (27) was most
275 likely first described by Galileo Galilei in 1638 . In order to calculate the length of the free
276 septal edge, which were newly formed in addition to the isometric scaled growth of the lung,
277 we mathematically corrected the growth induced decrease of the length density by
278 multiplying the length density by a factor of $\sqrt[3]{(V_x/V_0)^2}$ (thereby V_x represents the
279 parenchymal lung volume at the time point X, and V_0 the volume at the start of the growth).
280 The resulting “growth corrected length density” stays constant throughout isometric scaled
281 growth of the lung parenchyma, but shows an increase if new septa are formed. Therefore,
282 the increase of the growth corrected length density was taken as a measure for the anlage of
283 new alveolar septa as follows. The growth corrected length density at a given day was
284 divided by the growth corrected length density at day 4 and multiplied by 100 to express the
285 result as percentage. Therefore, the anlage of new septa is given as increase of the septa
286 present at day 4.
287 The number of proliferating or TUNEL-positive cells as well as the total number of
288 cells was estimated using the physical disector principle (33, 76). The disector was kept
289 constant at 9 μm.
290
291 **Electron microscopical morphometry**
292 The fraction of the alveolar surface area characterized by a single or double layered
293 capillary network or an atypical appearance with more than two capillary layers was

294 estimated by intersection counting (33, 82). In addition, the thickness of the septum was
295 measured perpendicular to the surface of the septum at each intersection. Intersections with
296 lung epithelium adjacent to non-parenchymal structures were not taken into account (82).
297
298 **Synchrotron radiation x-ray tomographic microscopy and visualization**
299 5 samples of each time-point were scanned at the TOMCAT (X02DA) beamline at the
300 Swiss Light Source (SLS) of the Paul-Scherrer-Institute (PSI), Villigen, Switzerland (75). The
301 energy was tuned to 12.398 keV (corresponding to an x-ray wavelength of 1 Å). After
302 penetration of the sample, x-rays were converted into visible light by a thin Ce-doped YAG
303 scintillator screen (Crismatec Saint-Gobain, Nemours, France). Projection images were
304 further magnified by diffraction limited microscope optics and finally digitized by a high-
305 resolution CCD camera (Photonic Science Ltd., East Sussex, UK), (74). The optical
306 magnification was set to 10x and on-chip binning was selected to improve the signal to noise
307 ratio, resulting in isotropic voxels of 1.4^3 μm^3 for the reconstructed images. For each
308 measurement, 1500 projections were acquired along with dark and periodic flat field images
309 at an integration time of 100ms each (30, 31, 42-44). Data were post-processed and
310 rearranged into flat field corrected sinograms online. Reconstruction of the volume of interest
311 was performed on a 24-node Linux PC farm using highly optimized filtered back-projection
312 routines. We used a global thresholding approach for surface rendering. For 3D-visualization
313 and surface rendering we used the software Imaris (Bitplane AG, Zürich, Switzerland) on an
314 Athlon 64 3500 based personal computer. To enhance the contrast between air and lung
315 tissue and to smooth the images we applied the gamma correction tool using the software
316 Adobe Photoshop C53 version 10.0 (Adobe Systems Incorporated, Microsoft Windows
317 Media Technologies).
318
319 **Statistical analysis**
320 The Kolmogorov-Smirnov test was applied to assess the Gaussian distribution of the
321 data. Differences between groups were assessed by one-way analysis of variance (ANOVA)

followed by Bonferroni-Holm-corrected post hoc t-tests (2, 57). Statistical significance was defined as $\alpha<0.05$. For all morphometrical measurements 3-8 male animals per time point were used (see figure legends).

Results

Morphological observation in 3D visualizations of the lung parenchyma

In order to study the effect of TNC deficiency during postnatal lung development, we morphologically compared the three-dimensional (3D) structure of the terminal airspaces between TNC null and wildtype lungs during the phase of classical alveolarization. As method, we used 3D- visualizations which were obtained by x-ray-tomographic microscopy. At day 4 the lungs of TNC deficient and of wildtype animals showed a similar appearance. The lung parenchyma was characterized by large terminal airspaces (saccules) in both groups (Fig. 1A+B). At day 7 newly formed septa and alveoli were detected in wildtype lungs indicating that alveolarization is ongoing (Fig 1D). TNC null lungs of the same age appeared to be in a state analogous to day 4, but showed focal areas of atypically thickened septa (Fig. 1C, arrowhead). At postnatal day 15 we were not able to observe any structural differences between both groups by morphological inspection of the 3D-visualizations at light microscopical resolution (Fig. 1E+F).

Stereological estimations

To verify our observations the lung volumes, the anlage of new alveolar septa and the septal surface area were quantified and compared between wildtype and TNC null animals. The lung volumes of TNC deficient animals were increased by 10-20% between days 2-21 (Fig. 2A). We did not observe any differences regarding the body weight of TNC null versus wildtype animals. Thus, the specific lung volume (lung volumes per body weight) of TNC null animals was larger than the one of wildtype mice between days 4-21 (data not shown). By following the anlage of new alveolar septa and alveolar surface area, we observed that the first and second phase of alveolarization (classical and continued alveolarization) was delayed. Alveolarization started delayed after day 7 in TNC deficient lungs, was compensated at days 15 -21, again delayed at day 36 and again compensated at day 60 and afterwards (Fig. 2 B+C).

By comparing the morphology of the interairspace septa between wildtype and TNC null lungs using light- and electron microscopy, focal areas of atypically thickened septa with an accumulation of capillaries and connective tissue, as well as an increased cellularity were observed in TNC null lungs at day 7 (Fig. 3). To better characterize this phenotype [i] the septal wall thickness was measured, [ii] microvascular maturation was followed by estimating the septal surface area possessing double versus single layered capillary networks on electron-microscopical images using intersection counting, and [iii] the number of proliferating cells (Ki-67-positive cells) as well as [iv] the total number of cells were stereologically estimated. The mean septal wall thickness of TNC null lungs was increased by 100% at day 7 (Fig. 4A). A histogram of the measured thickness revealed a shift from the classes of thinner measurements (0-10 μm) to thicker measurements (15-65 μm) in the TNC null lungs at this age (Fig. 4B). This result underlines our impression that only focal areas of the septa are thickened. Microvascular maturation was delayed and started after day 7 in TNC null animals (Fig. 5). In addition, about 33% of the alveolar septa of the TNC null lungs showed an atypical appearance with more than two capillary layers at day 7. This phenotype was only observed in tenascin-C null lungs of this age. At days 15 and 21 microvascular maturation appeared to be overcompensated. The percentage of mature septa was increased in TNC null lungs, but the difference disappeared at day 36. At day 60 a decreased fraction of mature septa was detected in TNC null mice. In adult animals at day 86 no differences were observed (Fig. 5). Following cell proliferation by estimating the number of Ki-67-positive cells, a peak of proliferating cells was detected at days 4 and 6 both in wildtype and TNC null animals. However, the number of proliferating cells was significantly larger in tenascin-C null lungs than in wildtype (Fig. 6C). The total number of cells was increased in tenascin-C deficient lungs at days 10 and 14, but at day 17 or later no differences were observed (Fig. 6D and data not shown).

Asking whether this disappearance of the difference in the total normal number of cells at day 17 or later may be explained by an increased rate of cell death, the number of TUNEL-positive cells was compared between TNC null and wildtype lungs. The TUNEL-

essay stains cell possessing a large amount of DNA breakage which is typical for apoptosis, programed cell death, and highly elevated DNA repair. Both in wildtype and TNC null animals a peak of TUNEL-positive cells was observed at days 14 and 17. In addition, TNC deficient lungs showed a premature increased rate of TUNEL-positive cell at day 10. At all other investigated time points no differences were observed (Fig. 7).

Discussion

Although numerous reports on TNC expression during organ and tissue development exist and many in vitro studies have suggested multiple functions for this protein during development (see introduction and (36) (9)), until now only one developmental abnormality has been reported in lungs of TNC deficient mice (60) (14). Recently we described a reduced branching morphogenesis during the development of the bronchial tree (60). In the present study we investigated the effect of TNC deficiency during postnatal lung development using the TNC null mice strain of Forsberg et al. (24). Early postnatal lung development is characterized by the start of alveolarization and microvascular maturation as well as a peak of cell proliferation. It is well acknowledged that TNC expression peaks in the lung while these processes take place. By following the anlage of new septa and microvascular maturation by stereological estimations, we observed that both developmental processes were delayed in TNC null lungs and started after day 7 (Figs. 2B, 5A, and 8) which is 3-4 days too late. This result lets us conclude that TNC contributes to the regulation of alveolarization/septation and microvascular maturation during early postnatal lung development. Remarkably, at day 7 about one third of the septal surface area present in TNC null lungs showed an atypical structure with more than 2 capillary layers (Fig. 5B). To the best of our knowledge such incorrectly structured septa have never been detected during postnatal lung development before.

TNC and cell migration

The expression of TNC is in parts controlled by mechanical stimuli. Furthermore, the presence of TNC facilitates cell migration e.g. by binding of TNC to the cell binding domain of fibronectin or by the recognition of TNC by α8 integrin (8, 13, 15, 78). Since the process of lifting off of new alveolar septa most likely includes mechanical forces and requires a coordinated migration of all cell types present in the distal lung, this phenotype implies that cell migration and the transduction of mechanical forces may be impaired by TNC deficiency. In consequence, we hypothesize that TNC contributes to mechano transduction and to the

415 regulation of cell migration which are both required for the lifting off of new alveolar septa and
416 for the formation of correct structured alveolar septa including the capillary network during
417 early postnatal lung development.
418
419 **Cell proliferation**
420 In parallel to the delayed start of alveolarization and microvascular maturation, an increased
421 number of proliferating cells was detected in TNC deficient lungs at days 4 and 6 (Fig. 6C).
422 We therefore conclude that TNC also takes part in the regulation of cell proliferation, and
423 thus seems to be a key factor for the regulation of the major developmental processes, i.e.
424 alveolarization, microvascular maturation, cell migration and cell proliferation, taking place
425 during early postnatal lung development. Moreover, our result of an increased cell
426 proliferation is basically interesting, because this study is the first one detecting this
427 phenomenon in vivo in TNC deficient mice. Many *in vitro* studies have shown that depending
428 on the cell type TNC can either stimulate (37) (11) (35) (72) (16) or inhibit cell proliferation
429 (11) (55) (17). However, *in vivo* studies have only observed a reduced rate of cell
430 proliferation in TNC deficient mice so far, namely in association with a model of renal
431 glomerulonephritis and in association with the behavior of neural precursor cells during the
432 development of the central nervous system (28) (51). Thus, our results indicate that TNC can
433 exert supportive or inhibitory effects on cell proliferation not only in vitro, but also in vivo.
434
435 **Mechanical forces**
436 Taking into account that TNC expression may be induced due to mechanical strain, and TNC
437 is highly expressed at the tips of the alveolar septa, which are recognized to bear high
438 mechanical forces, we moreover speculate that TNC expression might be up-regulated by
439 mechanical stimuli during early postnatal lung development. Since TNC seems to contribute
440 to the regulation of cellular processes like cell migration and cell proliferation during early
441 postnatal lung development, its function during this period might be described as mechano-

442 transducer, in a sense that mechanical stimulation promotes cellular action via up-regulation
443 of TNC expression.
444
445 **Continued alveolarization**
446 The peak of TNC expression during the first postnatal week is followed by a decline to
447 markedly reduced, but still detectable, levels during the third postnatal week during normal
448 lung development in rats and mice (58, 63, 85). Unexpectedly, besides impairing early
449 postnatal lung development (classical alveolarization), TNC deficiency did also alter later
450 stages of lung development. In TNC deficient lungs the continued alveolarization (second
451 phase) was prolonged. While a premature microvascular maturation was detected at days 15
452 and 21 (Fig. 2B and 5A), a delay of microvascular maturation was observed at day 60. The
453 latter may be due to the catch-up of alveolarization observed between days 36 and 60 in the
454 TNC deficient lungs, because newly formed septa are immature and it takes a short while
455 until they mature. Therefore, we hypothesize that the observed higher “input rate” of new
456 septa induces a higher percentage of immature septa in the TNC deficient lungs at day 60.
457 The altered continued alveolarization let us hypothesize that TNC contributes not only to the
458 lifting off of new septa and microvascular maturation at the start of postnatal lung
459 development, but that the low levels of TNC detected during later stages of lung
460 development also contribute to the regulation of both of the latter named processes.
461
462 **Programmed cell death.**
463 Given the roles of the protein TNC both during early postnatal lung development and during
464 later stages which have been demonstrated in the present study, it seems to be surprising
465 that no differences were detected in TNC deficient lungs in adulthood at postnatal day 86.
466 This result may be explained by the presence of corrective mechanisms during postnatal
467 lung development in TNC deficient animals. Although an increased number of proliferating
468 cells was observed in TNC null lungs at days 4 and 6, the total number of cells was
469 increased only at days 10 and 14, but not thereafter (Fig. 6). A possible mechanism

explaining this phenomenon would be a compensatory alteration of the rate of programmed cell death similarly to the alterations observed in rat which were treated with dexamethasone as neonates (45). Estimating the number of TUNEL-positive cells a premature peak of positive cells was detected in TNC null animals at day 10 (Fig. 7). Unfortunately, the TUNEL-essay is not completely specific for programmed cell death. It also detects cells expressing a high amount of DNA breakage during DNA repair. Because, programmed cell death is reported during this stage of development, it is likely that at least a high number of the TUNEL-positive cells are dying. The present study is the second one reporting that both cell proliferation and programmed cell death are altered in TNC deficient mice during development. However, in contrast to our observation Garcion et al. found a *reduced* rate of cell proliferation and a *reduced* rate of programmed cell death of neural precursor cells during development of the central nervous system in TNC deficient mice (28). Thus, the compensation of impaired cell proliferation by the adaptation of programmed cell death seems to be an important corrective mechanism which leads to the apparent normality of TNC deficient mice in adulthood. Furthermore, it needs to be mentioned that the present study is the first one following TUNEL-positive cells in the developing mouse lung. Over the last decades postnatal lung development was generally considered to be identical in mice and rats. However, while rat lung development was well characterized by morphometrical methods, postnatal lung development in the mouse was only followed by morphological observations. We were recently able to show that postnatal lung development in mice and rats is not identical regarding the endpoint of alveolarization, the rate of the anlage of new alveolar septa and the growth rate of the lung parenchyma (70) (50). In the present study we further observe that the peak of TUNEL-positive cells starts earlier in mice than in rats. While in rats a peak was detected at days 19 and 21 (45) (68), the peak was observed in mice already at days 14 and 17 (Fig. 7).

Rescue of phenotype

497 An additional question arising is why the anlage of new alveolar septa and septal surface
498 area observed in adult TNC deficient animals is identical to wildtype. The phenotype of a
499 practically absent formation of new septa between days 4-7 in TNC deficient lungs was
500 compensated in the TNC deficient lungs leading to an increased rate of newly forming septa
501 between days 7-15. The reduced formation of new septa between days 21-36 in TNC
502 deficient animals was compensated by prolonged alveolarization between days 36-60 (Fig.
503 2B). In parallel to the prolonged alveolarization a decrease of the fraction of the septal
504 surface area characterized by a single layered capillary network (mature capillary network)
505 was observed between days 36-60 in TNC deficient lungs (Fig. 5A). Principally, the lifting off
506 of new septa requires the existence of a double layered capillary network. While during
507 classical alveolarization (first phase) double layered capillary networks are still present in the
508 prenatally formed septa, the late formation of new septa is facilitated by local duplications of
509 the single layered capillary network at the sides of septation (70) (65). Most likely the double
510 layered capillary networks which are present during adolescence and young adults in
511 wildtype and TNC deficient lungs (Fig. 5A) appear at sites where new septa are forming and
512 grow into the alveolar lumen. Therefore, it is anticipated that an increased rate of the
513 formation of new septa should result in a transient immaturity of the alveolar septa.
514 Another process which was associated with TNC deficiency during postnatal lung
515 development and which practically represents a rescue mechanism is the increased lung
516 volume we observed in TNC null animals during early postnatal lung development (Fig. 2A).
517 Although in TNC null lungs practically no septa were formed between days 4-7 and the
518 anlage of septa was still decreased at day 10, the septal surface area of TNC deficient
519 animals was only slightly reduced at day 7 and not significantly different at day 10 (Fig. 2C).
520 However, this compensatory effect was restricted to the first three postnatal weeks and did
521 not rescue the reduced formation of new alveolar septa between days 21-36.
522 In principle the increase of the lung volume could be caused by two different effects:
523 increased lung growth and/or increased compliance of the lung tissue and the thorax (the
524 filling of the lungs was done when the thorax was still closed except a small hole in the

525 diaphragm). We would not like to speculate which of the mechanisms is predominately
526 involved. However, for the TNC deficient mice it does not matter, because in both cases,
527 lung growth versus compliance, a larger gas-exchange area could be used as compared to
528 wildtype – and most likely the latter is what counts for the mice.
529 Does the question of increased lung growth versus lung compliance effect the
530 stereological estimations? No, because the estimations are done as good as possible under
531 standardized conditions. To use a constant pressure for filling represents the state of the art
532 (34). However, stereological estimations do not tell anything about the reason why a
533 parameter is different in different groups of animals.
534 Does the question affect the finding of delayed and catch-up alveolarization? No,
535 because without the observed increase of lung volume the effect would be even more
536 pronounced.
537
538 **Conclusion**
539 In summary, we describe a new developmental phenotype of the TNC null mouse. In TNC
540 deficient lungs both alveolarization and microvascular maturation started with a delay, cell
541 proliferation was increased and thick septa with an accumulation of capillaries and cells were
542 observed during early postnatal lung development. These results are summarized in figure 8.
543 These results let us hypothesize that TNC contributes to the lifting off of new septa, the
544 regulation of cell migration and cell proliferation, and furthermore to microvascular maturation
545 at the start of postnatal lung development. The increased cell proliferation was most likely
546 rescued by an increased number of dying cells (TUNEL-positive), while the delayed
547 alveolarization and microvascular maturation were compensated by an increase of the
548 formation of new alveolar septa and an increase of septal maturation. In addition, the phase
549 of continued alveolarization (second phase) was prolonged and in parallel the alveolar
550 microvascular was less mature in TNC deficient mice towards the end of continued
551 alveolarization. The latter may be explained by an increased or better catch-up formation of
552 new alveolar septa which are immature directly after they are formed. We hypothesize that

TNC contributes not only to the lifting off of new alveolar septa and microvascular maturation during early postnatal lung development, but also during later stages.

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Disclosures

The author(s) do not have to declare any kind of conflicts of interests.

Author’s contributions

In collaboration S.I.M. and J.C.S. performed all experiments including the handling of the animals and the harvesting or the lungs, analyzed the data, interpreted results of experiments, and prepared the figures. S.I.M. did the stereological counting and drafted the manuscript. J.C.S. conceived and designed the research project.

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809

Figures legends

Figure 1. 3D-visualizations of the terminal airspaces. On postnatal day 4 the lung parenchyma of wildtype (WT) and TNC null mice (TNC) consisted of large terminal airspaces (A+B). In wildtype mice the start of alveolarization is characterized by the formation of new septa (arrow) and alveoli (asterisk) on day 7 (D). In TNC null lungs focal areas of atypical thickened septa were detected (arrowhead in C) on day 7 which are indicative for a halted alveolarization. At postnatal day 15 the differences disappeared (E+F). Bar, 50μm; visualizations are based on synchrotron-based x-ray-tomographic microscopy.

Figure 2. Lung volume, total length of the free septal edge, anlage of newly forming alveolar septa, and total surface area. The lung volumes (A), the total length of the free septal edge (B), the anlage of newly forming alveolar septa (C), and the septal (alveolar) surface area (D) were stereologically estimated. The anlage of newly formed septa is normalized to day 2 and equal to the newly formed length of the free septal edge. The lung volumes of TNC null animals were increased by approximately 20% between days 2-21 (A). In TNC null lungs classical alveolarization started delayed after day 7 (B-D). Furthermore, the formation of new alveolar septa was markedly reduced between days 21-36, but alveolarization was prolonged to day 60 (B+C). The septal surface area of TNC deficient animals was decreased at days 7 and 36 (D). Error bars indicate the standard deviations; N = 5-8 lungs of male mice per time point and genotype.

Figure 3. Morphological observations on light and electron- microscopical images at postnatal day 7. The morphological appearance of the interairspace septa of wildtype and tenascin C null lungs was compared on light- (A+B) and electron microscopical images (C+D). We observed focal areas with atypical thickened septa in TNC null lungs at postnatal day 7 (arrows). The focally thickened areas showed an abnormal structure with an accumulation of capillaries and connective tissue, as well as an increased cellularity, but no epithelial cells inside the thickening. Bar, 50 μm in A+B; 20μm in C+D.

838

839 **Figure 4. Thickness of septa**. The thickness of the septa was measured on electron
840 microscopical lung images as shown in figure 3C+D. At day 7 the mean septal wall thickness
841 of the tenascin C null mice was increased by 100% as compared to wildtype mice (A). Panel
842 B shows a histogram of the thickness measured at postnatal day 7 using a class width of
843 5µm. In TNC deficient lungs a broader distribution and a shift to thicker septa was observed.
844 Error bars indicate the standard deviations; N = 5 lungs of male mice per time point and
845 genotype.

846

847 **Figure 5. Microvascular maturation.** In TNC null lungs microvascular maturation was
848 delayed and started after day 7 (A). About one third of the alveolar septa of TNC deficient
849 animals showed an atypical appearance with more than two capillary layers at day 7 (B). At
850 days 15 and 21 premature microvascular maturation was detected. The difference
851 disappeared at day 36. At day 60 a decreased fraction of single layered septa was observed
852 in TNC null lungs, but in adult animals at day 86 no differences were detected (A). Error bars
853 indicate the standard deviations; N = 5 lungs of male mice per time point and genotype.

854

855 **Figure 6. Cell proliferation and total number of cells.** Lung sections of TNC deficient and
856 wildtype mice were stained with anti-Ki-67, a marker for cell proliferation, and counterstained
857 with hematoxylin as shown for postnatal day 6 (A+B). The number of Ki-67- positive cells (C)
858 as well as the total number of cells (D) per cubic millimeter of septal volume was evaluated
859 between postnatal days 4-17. Both in wildtype and in TNC null lungs a peak of proliferating
860 cells was detected at days 4 and 6, but the number of Ki-67- positive cells observed in mice
861 lacking TNC exceeded the one in wildtype (C). At days 10 and 14 the total number of cells
862 per cubic millimeter was larger in TNC null than in wildtype lungs (D). Bar, 50 µm. Error bars
863 indicate the standard deviations; N = 3 lungs of male mice per time point and genotype.

864

Figure 7. TUNEL-essay positive cells. Sections of TNC null and wildtype lungs were labelled by the TUNEL-procedure as shown for postnatal day 10 (A+B). The TUNEL-essay stains cell possessing a large amount of DNA breakage which is typical for programed cell death and highly elevated DNA repair. A peak of TUNEL-positive cells was observed at days 14 and 17 both in TNC deficient and wildtype lungs. At day 10 a four-fold increase was detected in lungs lacking TNC as compared to wildtype (C). Bar, 200 μm. Error bars indicate the standard deviations; N = 3 lungs of male mice per time point and genotype.

Figure 8. Timeline of phenotypes. The different phenotype in TNC deficient lungs are compared to the phenotype of wildtype lungs. **Panel A** summarizes the structural differences: Lung volumes of TNC deficient lungs are increased between days 4-21, but the length of the free septal edge, the anlage of septa and the alveolar surface area are all decreased at days 7 (-10) and 36. The three of them catch up at days 15-21 and a second time at day 60. **Panel B** illustrates cell number, proliferation, and death. An increased cell proliferation at days 4-7 is associated with an increased number of cells at days 10-15 in TNC deficient lungs. Programmed cell death is increased at day 10 which results in a normalization of the number of cells at days 15-86 as compared to wildtype. **Panel C** compares septal thickness and microvascular maturation. It seems to be that the delay of classical alveolarization at day 7 causes an increase of the septal thickness. Microvascular maturation is also effected at day 7, but showed a decreased maturity. The same was observed at day 60 which is most likely due to a catch-up alveolarization between days 36-60. All data are given as increase or decrease in comparison to wildtype (WT).